

- Inouye, Y. & Noda, M. (1955). *J. agric. chem. Soc. Japan*, **19**, 214.
Johansen, P. G., Marshall, R. D. & Neuberger, A. (1960). *Biochem. J.* **77**, 239.
Marshall, R. D. & Neuberger, A. (1961). *Biochem. J.* **78**, 31P.
Mills, G. L. (1952). *Biochem. J.* **50**, 707.

- Narita, K. (1958). *Biochim. biophys. Acta*, **28**, 184.
Ohno, K. (1953). *J. Biochem., Tokyo*, **40**, 621.
Phillips, D. M. P. (1961). *Biochem. J.* **80**, 40P.
Satake, K. & Seki, T. (1950). *J. Jap. Chem.* **4**, 557; cited from *Chem. Abstr.* (1951) **45**, 4604f.
Uraki, Z., Terminiello, L., Bier, M. & Nord, F. F. (1957). *Arch. Biochem. Biophys.* **69**, 645.

Biochem. J. (1963) **86**, 401

The Effect of the Administration of Carbon Tetrachloride on the Formation of Plasma Lipoproteins in the Rat

By ANNE SEAKINS AND D. S. ROBINSON

External Staff of the Medical Research Council, Sir William Dunn School of Pathology, Oxford

(Received 7 August 1962)

The subcutaneous injection of ethionine in the rat results in the development of a fatty liver, which is characterized by a specific rise in the concentration of triglyceride in the liver, and in a reduction in the concentration of all the neutral lipids of the plasma (Harris & Robinson, 1961). Soon after the ethionine has been given there is a marked fall in the rate of formation in the liver of the protein moiety of the plasma lipoproteins. Lipids are normally carried from the liver in combination with these proteins and it has been suggested, therefore, that this reduction in the rate of their formation will result in a failure to remove lipid from the liver at a normal rate and may hence be the cause of the subsequent changes in the concentrations of lipid in the liver and plasma (Robinson & Harris, 1961). A comparable inhibition of the formation of plasma-lipoprotein protein precedes the development of a fatty liver in the rat after the administration of the antibiotic, puromycin (Robinson & Seakins, 1962).

The fatty liver caused by the administration of carbon tetrachloride in rats is believed to be associated with a block in the passage of triglyceride from the liver to the plasma (Recknagel, Lombardi & Schotz, 1960; Recknagel & Lombardi, 1961; Heimberg & Weinstein, 1962). The possibility that inhibition of the formation of plasma lipoprotein occurs has been investigated in the present study. A preliminary account has been published (Robinson & Seakins, 1961).

METHODS

Female albino rats (Wistar strain) weighing 180–200 g. were used. They were starved for 16 hr. before being given, by stomach tube under light ether anaesthesia, either 1 ml. of a 1:1 (v/v) mixture of carbon tetrachloride and olive oil (test groups) or 0.5 ml. of olive oil (control groups).

Incorporation of DL-[1-¹⁴C]leucine into the proteins of rat liver and plasma

Incorporation in vivo. Rats were injected by the tail vein with 20 μ C of DL-[1-¹⁴C]leucine (7.2 μ C/m-mole) 1 hr. after they had been given either carbon tetrachloride and olive oil or olive oil alone. Then, 1.5 hr. after the injection, each animal was bled from a cannula in the abdominal aorta into 0.1 vol. of 3.8% (w/v) sodium citrate. The plasma was separated by centrifuging and stored at 0° until all the rats had been killed. The liver of each animal was removed, rinsed in 0.9% NaCl, dried on filter paper and weighed. A sample (about 0.5 g.) of each liver was homogenized immediately with 5 ml. of 10% (w/v) trichloroacetic acid and the homogenate was stored at 0°. When all the rats had been killed the liver homogenates were transferred to centrifuge tubes and the protein precipitates were aggregated in a boiling-water bath for 30 min. After being stored overnight at 0°, each precipitate was collected by centrifuging and repeatedly washed at 4° with 5% (w/v) trichloroacetic acid until the washings contained no detectable radioactivity. It was then extracted with three successive portions of hot acetone-ethanol (1:1, v/v) and once with diethyl ether.

The samples of citrated plasma were separated into protein fractions of d less than 1.063 (low-density lipoproteins), d 1.063–1.21 (high-density lipoproteins) and d greater than 1.21 (residue proteins) by high-speed centrifuging in the Spinco (model L) ultracentrifuge. After dialysis at 4° of the fractions against 0.9% NaCl adjusted to pH 7.4, they were transferred to centrifuge tubes, and ethanol and 20% (w/v) trichloroacetic acid were added to each, or to a suitable sample of the residue proteins, to give final concentrations of 70% (v/v) and 5% (w/v) respectively. The resulting protein suspensions were aggregated in a boiling-water bath for 30 min., stored overnight at 0° and centrifuged. The protein precipitates were washed with 70% (v/v) ethanol containing 5% (w/v) of trichloroacetic acid at 4° until the washings contained no detectable radioactivity, and were then extracted with three successive portions of hot acetone-ethanol (1:1, v/v) and one portion of diethyl ether.

Acid hydrolysis of the plasma-protein and liver-protein precipitates was carried out with 6N-HCl for 18 hr. at 105°. The hydrolysates were twice evaporated to dryness over P₂O₅ and solid KOH in a vacuum desiccator and the residue was dissolved in 0.5 ml. (plasma fractions) or 1 ml. (liver fractions) of citrate buffer, pH 5.0. After centrifuging, suitable samples of the supernatant solution were taken for the measurement of amino nitrogen and radioactivity.

Incorporation in vitro. Liver slices, prepared from the livers of test and control rats that had been given either carbon tetrachloride and olive oil or olive oil alone 2 hr. previously, were incubated at 37° under O₂ in 5 ml. of normal rat serum containing 8 μ C of DL-[1-¹⁴C]leucine (5.8 μ C/m-mole). After incubation for 4 hr. the serum samples were poured from the liver slices, centrifuged for 10 min. at 2500g to remove coarse tissue fragments and filtered through sintered-glass filters to remove any remaining liver particulate matter. The liver slices were rinsed in ice-cold Hanks's (1948) medium, dried and weighed. The preparation of acid hydrolysates of the liver proteins, the serum low-density lipoproteins, the serum high-density lipoproteins and the serum residue proteins was as described for the studies *in vivo*.

Changes in the concentration of free amino acids in the liver slices and the serum medium occurred during incubation. With liver slices weighing initially 160–240 mg., the tissue concentration, expressed as μ moles of free amino acid/100 mg. initial wet wt. of liver slice, fell from 0.97 ± 0.20 to 0.35 ± 0.25 in the test series and from 1.14 ± 0.21 to 0.59 ± 0.26 in the control series. In the serum, the concentration rose from 3.1 μ moles/ml. initially to 5.4 ± 0.8 μ moles/ml. in the test series and to 4.9 ± 0.3 μ moles/ml. in the control series. Thus changes in the concentration of free amino acids during incubation were similar whatever the origin of the liver slices. The study was not sufficiently extensive to show whether or not small differences between test and control animals occurred either in the initial concentrations of free amino acids in the liver slice or in the loss of free amino acids from the liver during incubation.

An experiment was carried out to determine whether the procedure used to aggregate the trichloroacetic acid precipitates, namely placing them for 30 min. in a boiling-water bath, resulted in any appreciable hydrolysis of the precipitated protein. With plasma fractions no additional free amino acids could be detected in the supernatant after boiling. With the liver fractions there was an increase in the free amino acids in the supernatant equivalent to 3% of the total liver-protein amino acid.

No radioactivity could be detected in acid hydrolysates when appropriate amounts of free DL-[1-¹⁴C]leucine were added, immediately before homogenizing in trichloroacetic acid, to livers or plasma-protein fractions from normal rats which were subsequently submitted to the procedures described above.

Incorporation of [³²P]orthophosphate into the phospholipids of rat liver and plasma

Incorporation in vivo. Rats were injected intraperitoneally with 6 μ C of [³²P]orthophosphate 15 min. after they had been given either carbon tetrachloride and olive oil or olive oil alone. Then 2.75 hr. later each rat was bled. The plasma was separated by centrifuging and stored at 0° until all the rats had been killed. The liver of each animal

was removed, rinsed in 0.9% NaCl, dried on filter paper and weighed. Separate samples (about 0.5 g.) were homogenized immediately with either 10 ml. of acetone-ethanol (1:1, v/v) or 10 ml. of ice-cold 5% (w/v) trichloroacetic acid.

Each liver homogenate in acetone-ethanol was boiled, cooled and filtered, and the precipitate on the filter paper was washed three times with 10 ml. of acetone-ethanol (1:1, v/v). The total filtrate was evaporated to dryness *in vacuo* at 55° on a rotary evaporator under N₂ and the residue was extracted three times with 20 ml. of hot diethyl ether. The total ether extract was washed with 40 ml. portions of 0.1M-NaCl in 0.1N-HCl until the washings contained no detectable radioactivity and then concentrated to a final volume of 5 ml. Samples were taken for counting and for the determination of total phosphorus.

The liver homogenates in 5% (w/v) trichloroacetic acid were centrifuged and samples of the supernatant solutions, containing acid-soluble phosphorus, were taken for counting and for the determination of total phosphorus.

The samples of citrated plasma were fractionated into low-density lipoproteins, high-density lipoproteins and residue proteins as described above. No radioactivity could be detected in lipid extracts of the residue proteins, and such fractions were not examined further. Each of the lipoprotein fractions was dialysed against 0.9% NaCl at pH 7.4 and 4° and then extracted with 50 ml. of acetone-ethanol (1:1, v/v). Subsequent treatment of the acetone-ethanol extracts was as described for the liver extracts.

Incorporation in vitro. Liver slices, prepared from the livers of test and control rats that had been given either carbon tetrachloride and olive oil or olive oil alone 2 hr. previously, were incubated at 37° under O₂ in 5 ml. of normal rat serum containing 5 μ C of [³²P]orthophosphate. In some experiments the serum medium was supplemented with sodium pyruvate (4 mM), sodium fumarate (1 mM), sodium glutamate (4 mM) and glucose (15 mM). After incubation for 4 hr. the serum was poured off from the liver slices, centrifuged and filtered through sintered-glass filters. Potassium bromide was added to the filtrate to raise the serum density to 1.21 and, after high-speed centrifuging in the Spinco (model L) ultracentrifuge, the lipoproteins of *d* less than 1.21 were separated by slicing the Lusteroid centrifuge tube 2.1 cm. from the bottom of the tube cap (Robinson & Harris, 1961). The efficiency of the fractionation was confirmed by demonstrating that the 1 ml. of solution in the tube immediately below the recovered lipoprotein fraction contained no radioactive phospholipid. Lipid extracts of the lipoprotein fractions of *d* less than 1.21 were prepared as described for the studies *in vivo*. Only three washings of the ether extract with 0.1M-NaCl in 0.1N-HCl were required.

The efficiency with which free [³²P]orthophosphate was removed from the lipid extracts by the procedures described was determined. No contamination of lipid extracts of liver and plasma samples from normal rats could be detected when 5 μ C of [³²P]orthophosphate was added immediately before homogenizing with acetone-ethanol.

Incorporation of sodium [1-¹⁴C]acetate into the free cholesterol of rat liver and plasma in vivo

Rats were injected by the tail vein with 10 μ C of sodium [1-¹⁴C]acetate (8.1 mc/m-mole) 2 hr. after they had been given either carbon tetrachloride and olive oil or olive oil

Table 1. *Effect of the administration of carbon tetrachloride on the total esterified fatty acids, phosphatides and cholesterol of rat plasma and liver*

Female rats were fed with 1 ml. of a mixture of carbon tetrachloride and olive oil (1:1, v/v) (carbon tetrachloride-treated group) or 0.5 ml. of olive oil (control group). At 24 hr. they were killed by bleeding from the abdominal aorta and the amounts of lipids in the plasma and liver were determined.

	Rat no.	Wet wt. of liver (g.)	Total esterified fatty acids		Total cholesterol		Total phosphatides	
			Plasma (mg./ml.)	Liver (mg./whole liver)	Plasma (mg./ml.)	Liver (mg./whole liver)	Plasma (mg./ml.)	Liver (mg./whole liver)
Carbon tetrachloride-treated group	1	6.2	0.64	610	0.29	28	0.50	169
	2	7.4	0.60	612	0.27	32	0.51	192
	3	6.4	0.52	455	0.33	30	0.54	187
	4	7.0	0.54	600	0.39	31	0.61	204
Means		6.8	0.58	569	0.32	30	0.54	188
Control group	1	7.0	1.29	202	0.59	24	1.04	196
	2	6.8	1.52	196	0.58	22	0.93	198
	3	6.3	1.24	192	0.64	22	1.08	181
	4	7.2	1.42	193	0.61	21	1.08	206
Means		6.8	1.37	196	0.61	22	1.03	195

alone. At 0.5, 1 or 2 hr. after the injection, blood was collected from appropriate groups of rats into 0.1 vol. of 3.8% (w/v) sodium citrate and the livers were removed, rinsed in 0.9% NaCl and immediately homogenized in 100 ml. of acetone-ethanol (1:1, v/v). The homogenates of livers from individual rats were combined into test and control groups taken either 0.5, 1 or 2 hr. after the injections with sodium acetate. The plasma samples from individual rats were combined into similar groups and each grouped batch of plasma was blown into 25 vol. of acetone-ethanol (1:1, v/v).

The suspensions of liver proteins and of plasma proteins in acetone-ethanol were boiled, cooled and filtered, and the residues on the filter papers were washed three times with 50 ml. portions of hot acetone-ethanol (1:1, v/v). The filtrates were evaporated to dryness *in vacuo* at 55° on a rotary evaporator under N₂ and the residues taken up in either 150 ml. (liver residues) or 25 ml. (plasma residues) of acetone-ethanol (1:1, v/v). Free cholesterol was precipitated from the acetone-ethanol extracts as its digitonide according to the method of Sperry & Webb (1950). A weight of digitonin was used which was approximately 25 times the weight of free cholesterol calculated to be present in the extracts. The digitonide was washed as described by Sperry & Webb and dried at 37°. Free cholesterol was liberated by dissolving the digitonide in 5 ml. of pyridine and heating the solution at 70° for 20 min. (Bergmann, 1940). Dry ether (25 ml.) was added, the digitonin precipitate which formed was filtered off, and the ether solution was washed several times with N-HCl to remove excess of pyridine.

The ether solutions of the free cholesterol from plasma were evaporated to dryness at this stage and the cholesterol was redissolved in a suitable volume of methanol to which a little ethyl acetate had been added. Samples of this solution were taken for counting and for the determination of cholesterol. The free cholesterol from liver was further purified through its dibromide by the procedure of Schwenk & Werthessen (1952). The purified cholesterol was dissolved

in a suitable volume of methanol to which a little ethyl acetate had been added and samples were taken for counting and for the determination of cholesterol.

Concentration of lipid in plasma and liver

Lipid extracts of plasma and liver for the determinations of esterified fatty acids and phosphatides were prepared as described by Harris & Robinson (1961). Cholesterol was determined either directly in plasma samples (0.2 ml.) or in the residue obtained after drying suitable samples of the lipid extracts of plasma and liver *in vacuo*.

Counting procedure and analytical methods

The counting procedure and the determinations of amine nitrogen, esterified fatty acids, acid-soluble phosphorus, lipid phosphorus and cholesterol were carried out as described by Harris & Robinson (1961) and Robinson & Harris (1961), except that the method of Henly (1957) was used to measure the concentration of cholesterol in plasma and in the lipid extracts of plasma and liver prepared for radioactive measurements.

RESULTS

Recknagel *et al.* (1960) found a fall in the concentration of triglyceride in the plasma in rats given carbon tetrachloride. A fall in the concentrations of phosphatides and cholesterol in the plasma, as well as in the concentration of esterified fatty acids in the plasma, after the administration of carbon tetrachloride is shown in Table 1. The mean concentration of plasma esterified fatty acid was lowered in the treated animals to 42% of the control values 24 hr. after the administration of carbon tetrachloride. The mean concentrations of cholesterol and phosphatides in plasma were each lowered to 52% of the control values. The mean

content of esterified fatty acid in the liver was increased threefold after the administration of carbon tetrachloride. The concentration of cholesterol in the liver was moderately increased but the phosphatide content was unchanged.

Effect of the administration of carbon tetrachloride on the incorporation of DL-[1-¹⁴C]leucine into the proteins of rat liver and plasma

When DL-[1-¹⁴C]leucine was injected into female rats 1 hr. after they had been given carbon tetrachloride and olive oil, the incorporation of ¹⁴C into the proteins of the liver and plasma was much less than in animals which had been given olive oil alone (Table 2). The reduction in incorporation appeared to be greater for the plasma-protein fractions than for the liver protein.

A reduced incorporation of ¹⁴C *in vitro* after the administration of carbon tetrachloride was also demonstrated when liver slices from control and carbon tetrachloride-treated rats were incubated in normal rat serum containing DL-[1-¹⁴C]leucine (Table 3). Under these conditions ¹⁴C is incorporated into the liver proteins and into those serum proteins and lipoproteins which are newly synthesized by the liver and released into the serum medium (Radding & Steinberg, 1960). The reductions in incorporation into the three serum protein fractions and into the liver proteins were all

significant at a 0.1% level of confidence as measured by the Behrens test of the significance of differences between two means (Fisher & Yates, 1957).

Effect of the administration of carbon tetrachloride on the incorporation of [³²P]orthophosphate into the phosphatides of rat liver and plasma

Table 4 shows the relative specific activities of the liver phosphatides and of the high-density-lipoprotein phosphatides of the plasma of groups of rats which were injected with [³²P]orthophosphate 15 min. after being given carbon tetrachloride and olive oil or olive oil alone and which were killed 2.75 hr. later. Incorporation of radioactivity was decreased, after the administration of carbon tetrachloride, in the phosphatides of liver and of the high-density lipoproteins of the plasma, the decreases being significant at 5% and 1% levels of confidence respectively. In this experiment washing of the low-density-lipoprotein fractions of plasma resulted in considerable losses of lipoprotein. The levels of radioactivity in lipid extracts of the washed fractions were, in consequence, very low and they have not been reported.

Incubation of liver slices from control and carbon tetrachloride-treated rats in normal rat serum containing [³²P]orthophosphate showed that the amount of ³²P incorporated *in vitro* into

Table 2. *Effect of the administration of carbon tetrachloride on the incorporation of DL-[1-¹⁴C]leucine into the proteins of rat liver and plasma in vivo*

Rats were fed as described in Table 1 and injected intravenously with 20 μ C of DL-[1-¹⁴C]leucine 1 hr. later. Each rat was killed 1.5 hr. after the injection and the incorporation of ¹⁴C into the proteins of liver and of plasma was determined. Radioactivity values are expressed as the means \pm S.D. Each group consisted of six rats.

	Radioactivity in protein hydrolysates (counts/min./100 μ g. of amino N)			
	Plasma lipoproteins of $d < 1.063$	Plasma lipoproteins of $d 1.063-1.21$	Plasma proteins of $d > 1.21$	Liver proteins
Carbon tetrachloride-treated group	318 \pm 72	242 \pm 73	52.6 \pm 7.7	78.4 \pm 22
Control group	3438 \pm 242	1894 \pm 110	314 \pm 82	362 \pm 43
Carbon tetrachloride-treated group as % of control group	9.3	12.7	16.7	21.6

Table 3. *Effect of the administration of carbon tetrachloride on the incorporation of DL-[1-¹⁴C]leucine into the proteins of rat liver and serum in vitro*

Rats were fed as described in Table 1. At 2 hr. the rats were killed and slices from each rat liver were incubated for 4 hr. at 37° under O₂ in 5 ml. of rat serum containing 8 μ C of DL-[1-¹⁴C]leucine. Incorporation of ¹⁴C into the proteins of liver and of serum was determined. Radioactivity values are expressed as the means \pm S.D. Each group consisted of five rats.

	Radioactivity in protein hydrolysates (counts/min./100 μ g. of amino N)			
	Serum lipoproteins of $d < 1.063$	Serum lipoproteins of $d 1.063-1.21$	Serum proteins of $d > 1.21$	Liver proteins
Carbon tetrachloride-treated group	7.2 \pm 3.4	8.8 \pm 3.6	3.7 \pm 1.2	423 \pm 59
Control group	136 \pm 60	70 \pm 13	29 \pm 7.9	622 \pm 48

Table 4. *Effect of the administration of carbon tetrachloride on the incorporation of [^{32}P]orthophosphate into the phosphatides of rat liver and plasma high-density lipoprotein in vivo*

Rats were fed as described in Table 1 and injected intraperitoneally with [^{32}P]orthophosphate ($6\mu\text{C}/\text{rat}$) 15 min. later. Each rat was killed 3 hr. after feeding and the incorporation of [^{32}P]orthophosphate into the phosphatides of liver and plasma high-density lipoproteins was determined. Radioactivity values are expressed as the means \pm s.d. of the ratios of the specific activities of the phosphatide phosphorus to that of the liver acid-soluble phosphorus. Each group consisted of five rats.

	Relative specific activity of phosphatide phosphorus	
	Plasma lipoproteins of $d\ 1.063-1.21$	Liver
Carbon tetrachloride-treated group	0.022 ± 0.010	0.10 ± 0.01
Control group	0.047 ± 0.014	0.13 ± 0.02

the serum phosphatides was not significantly changed by the administration of carbon tetrachloride (Table 5). Supplementing the serum medium with glutamate, fumarate, pyruvate and glucose did not appreciably affect the extent of incorporation of ^{32}P in either carbon tetrachloride-treated or control animals.

Effect of the administration of carbon tetrachloride on the incorporation of sodium [$1\text{-}^{14}\text{C}$]acetate into the free cholesterol of rat liver and plasma

The studies of Van Bruggen, Hutchens, Claycomb & West (1953) and of Loud & Bucher (1958) have shown that, after the injection of sodium [$1\text{-}^{14}\text{C}$]acetate in the rat, the specific activity of the free cholesterol of the liver rises rapidly and reaches a maximum within 20–30 min. There is, however, considerable variation from animal to animal in the time-course of the labelling and in the maximum specific activity (see also Gould, Bell & Lilly, 1959). In an attempt to minimize the effects of individual variation as well as to provide sufficient material for study, livers and plasma samples from groups of rats were combined in the experiment described below.

Groups of rats were injected with sodium [$1\text{-}^{14}\text{C}$]acetate 2 hr. after being given either carbon tetrachloride and olive oil or olive oil alone, and the specific activities of the free cholesterol of liver and of plasma were measured at various times after the injection. The results are shown in Table 6. At all time-intervals the specific activities were lower in the carbon tetrachloride-treated animals than in the control animals, although the differences were much less marked 2 hr. after the injection of

Table 5. *Effect of the administration of carbon tetrachloride on the incorporation of [^{32}P]orthophosphate into the phosphatides of serum lipoprotein in vitro*

Rats were fed as described in Table 1. At 2 hr. the rats were killed and slices from each rat liver were incubated for 4 hr. at 37° under O_2 in either 5 ml. of rat serum containing $5\mu\text{C}$ of [^{32}P]orthophosphate (Expts. 1 and 2) or 5 ml. of enriched rat serum containing sodium pyruvate (4 mM), sodium fumarate (1 mM), sodium glutamate (4 mM), glucose (15 mM) and [^{32}P]orthophosphate ($5\mu\text{C}$) (Expt. 3). Incorporation of [^{32}P]orthophosphate into the phosphatides of serum lipoprotein was determined. Radioactivity values are expressed as means \pm s.d. Each group consisted of either five (Expt. 1) or three (Expts. 2 and 3) rats.

	Specific activity of serum phosphatide (counts/min./ μg . of phosphatide P)		
	Expt. 1	Expt. 2	Expt. 3
Carbon tetrachloride-treated group	1.45 ± 0.34	1.89	2.15
Control group	1.72 ± 0.40	2.40	2.31

Table 6. *Effect of the administration of carbon tetrachloride on the incorporation of sodium [$1\text{-}^{14}\text{C}$]acetate into the free cholesterol of rat liver and plasma in vivo*

Rats were fed as described in Table 1. At 2 hr. the rats were injected by the tail vein with $10\mu\text{C}$ of sodium [$1\text{-}^{14}\text{C}$]acetate. Then 0.5, 1 or 2 hr. after the injection the rats were killed and the incorporation of ^{14}C into the free cholesterol of liver and of plasma was determined. The livers and plasma samples from each group of rats (six rats/group) were pooled.

	Time after injection of acetate (hr.)	Specific activity (counts/min./mg. of cholesterol)	
		Plasma	Liver
Carbon tetrachloride-treated group	0.5	5.6	3.3
	1.0	9	18
	2.0	19	20
Control group	0.5	19	20
	1.0	28	30
	2.0	26	27

acetate than at 30 min. In the control animals the specific activity of the free cholesterol of the plasma was similar to that in the liver throughout, presumably because of rapid equilibration of plasma and liver cholesterol (see Swell, Trout, Field & Treadwell, 1958). In the carbon tetrachloride-treated animals the relation of the specific activity of cholesterol in the plasma to that in the liver was more variable.

DISCUSSION

The present studies show that, within 1–2 hr. of the administration to female rats of a dose of carbon tetrachloride which produces at 24 hr. a fatty liver and a fall in the concentration of all the neutral lipids of the plasma, there is a marked

reduction in the incorporation of ^{14}C from DL-[1- ^{14}C]leucine into the liver proteins and into the plasma proteins and lipoproteins produced by the liver.

This evidence for a reduction in the synthesis of the proteins of liver and plasma as an early effect of the administration of carbon tetrachloride is in accordance with the results of Oberling & Rouiller (1956) and of Bassi (1960), who have shown with the electron microscope that the endoplasmic reticulum of the liver cell, the presumed site of protein synthesis, is damaged within 1 hr. of the administration of carbon tetrachloride, i.e. when the toxic agent is at its maximum concentration in the liver (Recknagel & Litteria, 1960).

The inhibition of the synthesis of plasma-lipoprotein protein after the administration of carbon tetrachloride is of particular interest since it may explain the subsequent changes in the concentrations of lipid in plasma and in liver. It seems reasonable to suggest that reduced synthesis of plasma lipoprotein will result in a fall in the amount of lipid which is normally transported from the liver in combination with this protein and that this will lead both to a decrease in the concentration of neutral lipids in the plasma and to the accumulation of fat in the liver. The failure of the 'triglyceride-secretory mechanism' of the liver, which Recknagel & Litteria (1960), Recknagel *et al.* (1960) and Recknagel & Lombardi (1961) found after the administration of carbon tetrachloride, can thus be attributed to the inhibition of the formation of plasma-lipoprotein protein. The possibility that carbon tetrachloride increases the synthesis of triglyceride in the liver (Judah & Rees, 1959) and that such an increase contributes to the rise in concentration of triglyceride in the liver is not excluded by these results.

If the mode of action of carbon tetrachloride in causing a fatty liver is as described above it appears to be analogous to that of ethionine, a methionine antagonist, and of the antibiotic, puromycin. The administration of each of these substances to rats results in the development of fatty livers associated with reductions in the concentration of neutral lipids in the plasma under conditions in which the incorporation of amino acids into the liver proteins and the plasma proteins and lipoproteins is markedly reduced (Harris & Robinson, 1961; Robinson & Harris, 1961; Robinson & Seakins, 1962).

Although the triglyceride moiety of the plasma lipoproteins is synthesized in the liver, a proportion of the component fatty acids do not originate in that organ but are transported there in the free form from the adipose tissue; the fatty acid composition of the triglycerides which accumulate in the liver after the administration of carbon tetra-

chloride indicates this origin in the fat depots (Horning, Earle & Maling, 1962). The phosphatide and cholesterol components of the plasma lipoproteins, on the other hand, are synthesized in the liver itself. These differences in the origin of the various components of plasma-lipoprotein lipid may explain why only triglyceride accumulates in the liver after the administration of carbon tetrachloride since, whereas the rates of synthesis or of destruction of phosphatide and cholesterol may be regulated by the liver itself, the inflow of free fatty acids from the plasma is probably determined by such factors as the rate of mobilization of fat from the depots which are outside the control of the liver.

Some evidence that a fall in the synthesis of plasma phosphatides accompanied the reduction in the synthesis of plasma-lipoprotein protein *in vivo* was obtained in the present studies. Thus a moderate reduction in the incorporation of ^{32}P into the phosphatides of the high-density lipoproteins of plasma occurred in animals injected with [^{32}P]orthophosphate shortly after they had been given carbon tetrachloride. On the other hand, no evidence for a reduced synthesis of the plasma phosphatides was obtained in the ^{32}P -incorporation studies with liver slices *in vitro* and it appears, therefore, that the reduction in the synthesis of lipoprotein protein, under these circumstances, was independent of any effect on the synthesis of plasma phosphatides.

The studies on the incorporation of injected sodium [1- ^{14}C]acetate into the cholesterol of plasma and liver were limited and are difficult to interpret. It is known from work with normal rats that the tracer doses of acetate which were used disappear from the circulation with a half-time of less than 1 min. (Busch & Baltrush, 1954) and that a further lag period of about 10 min. then ensues before labelled cholesterol appears in the liver (Loud & Bucher, 1958). This lag period represents the time taken for the injected acetate to pass through the various intermediaries in cholesterol biosynthesis. The results of the present study suggest that the administration of carbon tetrachloride may extend this lag period since, whereas, as was expected, the specific activity of the cholesterol of both liver and plasma was already high and close to its maximum in the control animals 30 min. after the acetate injection, it was much lower at this time in the animals that had been given carbon tetrachloride. By 2 hr. after the injection of the acetate, on the other hand, the specific activities of the cholesterol of liver and of plasma in the carbon tetrachloride-treated animals had risen to levels which were similar to those in the control animals.

In the preceding discussion, the isotope-incorporation experiments have been interpreted as suggesting that the administration of carbon tetra-

chloride causes a reduction in the synthesis of plasma-lipoprotein protein and that this may result in a secondary inhibition of the synthesis of the lipid components of the lipoprotein. An alternative explanation might be that the release of preformed lipoprotein molecules from the liver was prevented after the administration of carbon tetrachloride. This would require, however, that the incorporation of both [^{32}P]orthophosphate and [$1\text{-}^{14}\text{C}$]acetate into the plasma lipids should be diminished in proportion to the reduction in incorporation of ^{14}C from DL-[$1\text{-}^{14}\text{C}$]leucine into the plasma-lipoprotein protein. This was not so under the conditions of this investigation.

The almost complete inhibition after the administration of carbon tetrachloride of the incorporation of ^{14}C from DL-[$1\text{-}^{14}\text{C}$]leucine into the plasma-lipoprotein protein *in vitro* was accompanied by a less dramatic reduction of incorporation into the liver proteins themselves. A similar result was obtained with puromycin (Robinson & Seakins, 1962). If these changes are due to a reduction of protein synthesis they show that the inhibitory effects of carbon tetrachloride and of puromycin can vary with the particular proteins that are being synthesized.

SUMMARY

1. The administration of a mixture of carbon tetrachloride and olive oil to female rats led to the following changes at 24 hr. in the liver and plasma lipids: (a) a rise in the amount of esterified fatty acids in the liver from 196 to 569 mg.; (b) a rise in the amount of cholesterol in the liver from 21 to 30 mg.; (c) a fall in the concentration of esterified fatty acids in the plasma from 1.37 to 0.58 mg./ml.; (d) a fall in the concentration of cholesterol in the plasma from 0.60 to 0.32 mg./ml.; (e) a fall in the concentration of phosphatides in the plasma from 1.03 to 0.54 mg./ml.

2. At 2 hr. after the same dose of carbon tetrachloride the incorporation of DL-[$1\text{-}^{14}\text{C}$]leucine, injected *in vivo*, into the low-density lipoproteins, high-density lipoproteins and residue proteins of the plasma was respectively 9.3, 12.7 and 16.7 % of the incorporation into the corresponding fractions in control animals given olive oil alone. Incorporation into the liver protein was reduced to 21.6 % of the level in control animals. Liver slices from rats dosed with carbon tetrachloride, incubated *in vitro* in a serum medium containing DL-[$1\text{-}^{14}\text{C}$]leucine, showed similar reductions of incorporation into plasma proteins. The reduction of incorporation into the liver proteins was less marked.

3. A significant reduction of the incorporation of [^{32}P]orthophosphate into the phosphatides of

liver and of plasma was found *in vivo* 3 hr. after the administration of carbon tetrachloride. The incorporation of [^{32}P]orthophosphate into plasma phosphatides *in vitro* was not significantly affected 2 hr. after the administration of carbon tetrachloride.

4. At 0.5, 1 and 2 hr. after the injection of sodium [$1\text{-}^{14}\text{C}$]acetate the specific activities of the cholesterol of liver and of plasma were lower in animals given carbon tetrachloride 2 hr. previously than in control animals.

5. These results suggest that inhibition of the formation of plasma lipoprotein may be a cause of the fatty livers and of the lowered concentrations of plasma lipid in carbon tetrachloride-treated animals.

The authors thank Mrs S. Otway for her expert assistance.

REFERENCES

- Bassi, M. (1960). *Exp. Cell Res.* **20**, 313.
- Bergmann, W. (1940). *J. biol. Chem.* **132**, 471.
- Busch, H. & Baltrush, H. A. (1954). *Cancer Res.* **14**, 448.
- Fisher, R. A. & Yates, F. (1957). *Statistical Tables for Biological, Agricultural and Medical Research*, p. 57. Edinburgh: Oliver and Boyd Ltd.
- Gould, R. G., Bell, V. L. & Lilly, E. H. (1959). *Amer. J. Physiol.* **196**, 1231.
- Hanks, J. H. (1948). *J. cell. comp. Physiol.* **31**, 235.
- Harris, P. M. & Robinson, D. S. (1961). *Biochem. J.* **80**, 352.
- Heimberg, M. & Weinstein, I. (1962). *Biochem. Pharmacol.* **11**, 163.
- Henly, A. A. (1957). *Analyst*, **28**, 286.
- Horning, M. G., Earle, M. J. & Maling, H. M. (1962). *Biochim. biophys. Acta*, **56**, 175.
- Judah, J. D. & Rees, K. R. (1959). *Fed. Proc.* **18**, 1013.
- Loud, A. V. & Bucher, N. L. R. (1958). *J. biol. Chem.* **233**, 37.
- Oberling, Ch. & Rouiller, C. (1956). *Ann. Anat. path.* **1**, 401.
- Radding, C. M. & Steinberg, D. (1960). *J. clin. Invest.* **39**, 1560.
- Recknagel, R. O. & Litteria, M. (1960). *Amer. J. Path.* **36**, 521.
- Recknagel, R. O. & Lombardi, B. (1961). *J. biol. Chem.* **236**, 564.
- Recknagel, R. O., Lombardi, B. & Schotz, M. C. (1960). *Proc. Soc. exp. Biol., N.Y.*, **104**, 608.
- Robinson, D. S. & Harris, P. M. (1961). *Biochem. J.* **80**, 361.
- Robinson, D. S. & Seakins, A. (1961). *Biochem. J.* **82**, 9p.
- Robinson, D. S. & Seakins, A. (1962). *Biochim. biophys. Acta*, **62**, 163.
- Schwenk, E. & Werthessen, N. T. (1952). *Arch. Biochem. Biophys.* **40**, 334.
- Sperry, W. M. & Webb, M. (1950). *J. biol. Chem.* **187**, 97.
- Swell, L., Trout, E. C., Field, H. & Treadwell, C. R. (1958). *J. biol. Chem.* **230**, 631.
- Van Bruggen, J. T., Hutchens, T. T., Claycomb, C. K. & West, E. S. (1953). *J. biol. Chem.* **200**, 31.